RESEARCH ARTICLE

Figures and figure supplements

An in vitro stem cell model of human epiblast and yolk sac interaction

Kirsty ML Mackinlay *et al*

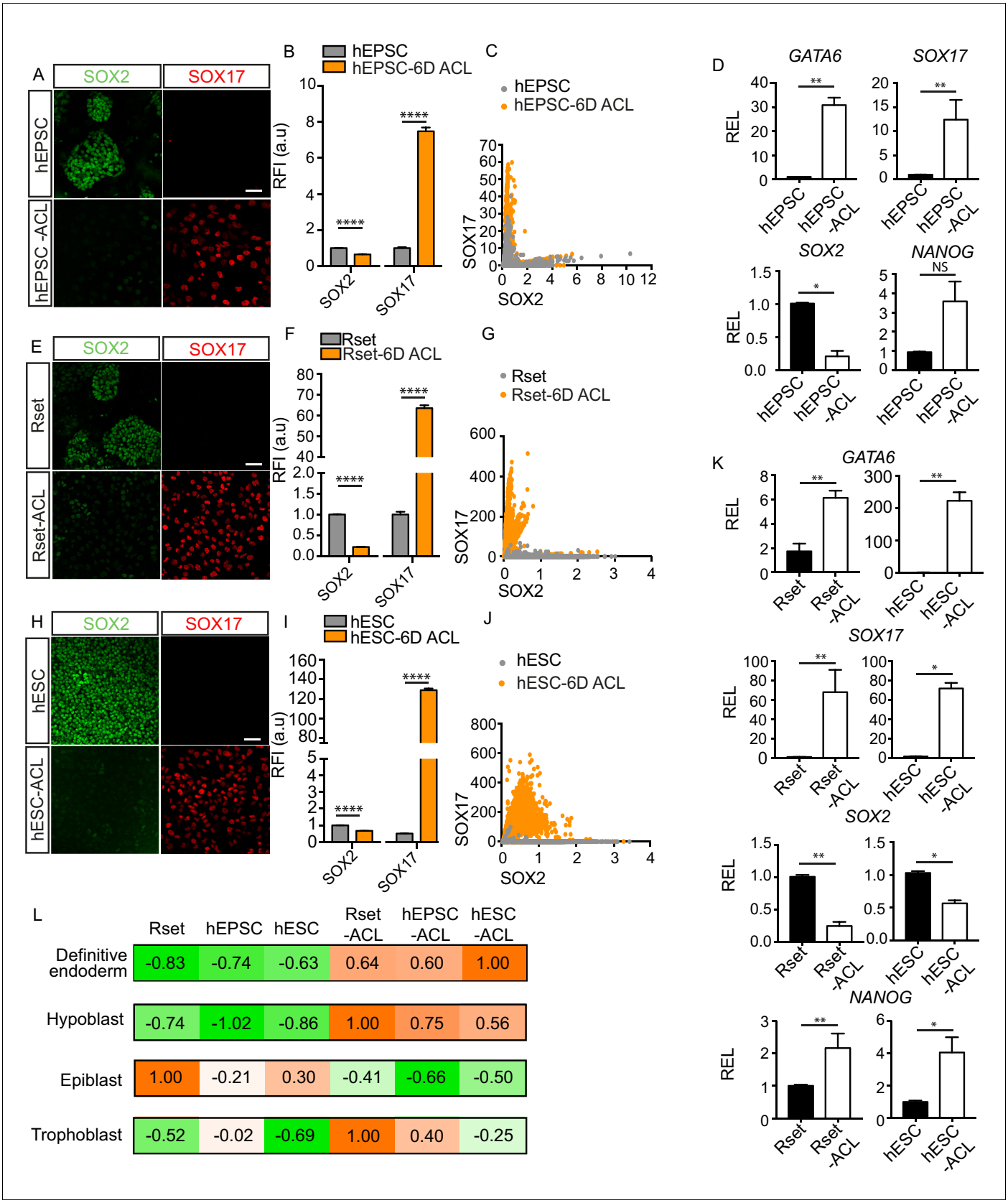

Figure 1. Endoderm conversion of RUES2-GLR human pluripotent stem cells (hPSCs) in response to ACL treatment. (**A, E, and H**) Immunofluorescence images of SOX2 (green) and SOX17 (red) in human extended pluripotent stem cells (hEPSCs), Rset human pluripotent stem cells (hPSCs), and human

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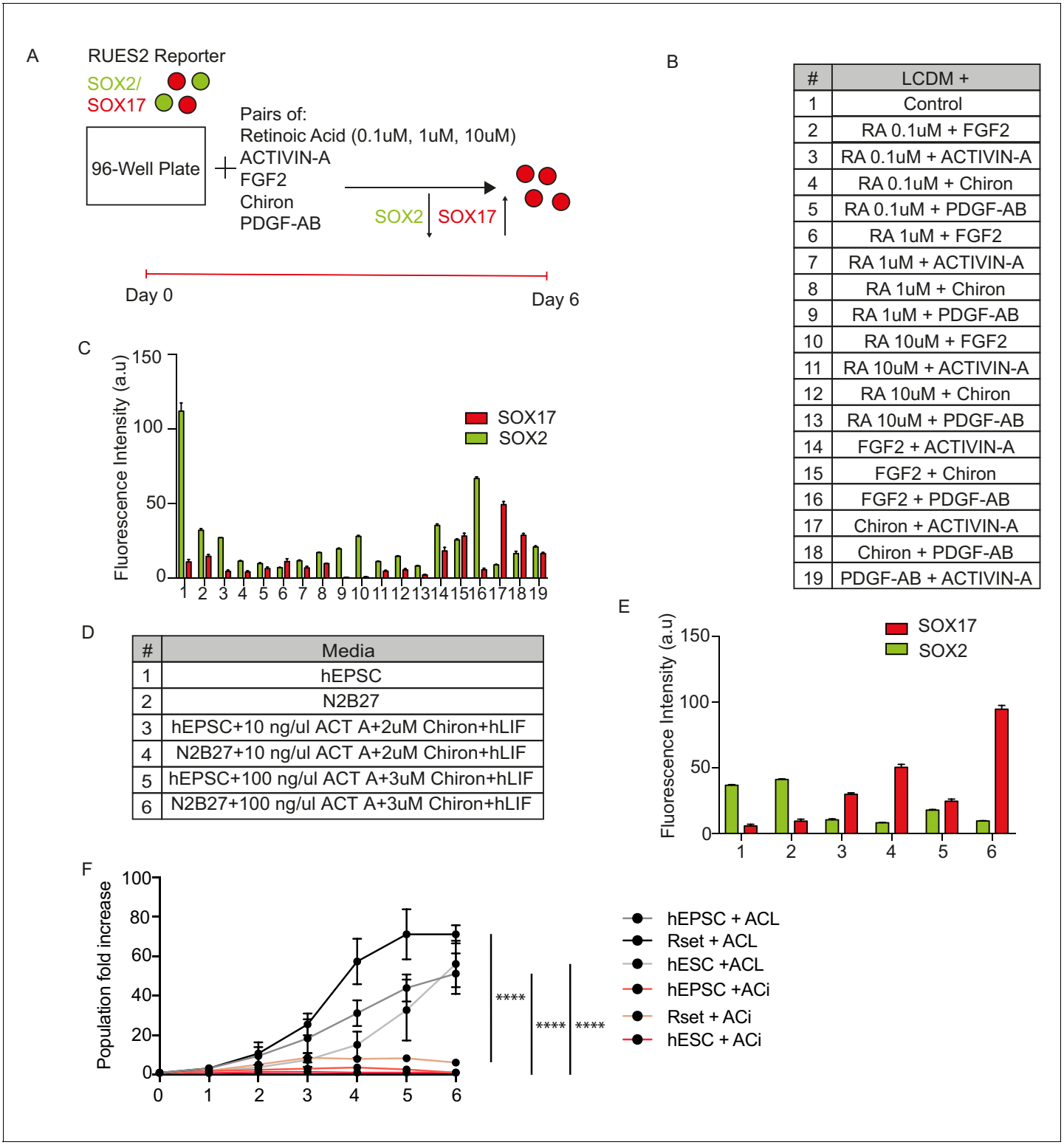
embryonic stem cells (hESCs) after 6 days (6D) of ACL treatment (scale bar = 50 μm). (**B, F, and I**) Bar chart of elative fluorescence intensity (RFI arbitrary units [a.u.]) of SOX17 and SOX2 (± SEM) before and after 6D of ACL treatment in hPSCs as measured via reporter protein immunostaining. Mann–Whitney U-test; ****p < 0.0001 (number of cells analysed: hEPSC control n=2736, hEPSC + 6D ACL n=2270, Rset control n=2749, Rset + 6D ACL n=3146, hESC control n=1939, hESC + 6D ACL n=2538, three independent experiments each with one sample). (**C, G, and J**) Quantification of SOX2 and SOX17 protein levels at the single cell level in hEPSC, Rset hPSC, and hESC 6D ACL-treated cells based on relative reporter fluorescence intensity. Each dot represents an individual cell. (**D**) Relative expression levels (REL) (± SEM) of *GATA6*, *SOX17*, *NANOG* (n=6 samples, three independent experiments) *and SOX2* (n=4 samples, two independent experiments) in hEPSC before and after 6D ACL treatment, normalised to their respective control. Mann–Whitney U-test *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (**K**) Relative expression levels (REL) (± SEM) of *GATA6*, *SOX17*, *SOX2*, and *NANOG* in Rset hPSCs (n=6 samples, three independent experiments), or hESCs (n=4 samples, two independent experiments) after 6D ACL treatment, normalised to their respective control . Mann–Whitney U-test *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (**L**) Transcriptomic-signature score (gene set variation analysis ([GSVA]) positive marker score) when comparing ACL-treated cells to either the human hypoblast, trophoblast, or epiblast within the human embryo (E6–14), or to human definitive endoderm using sc-RNA-seq expression data (embryo and definitive endoderm) and bulk RNA-seq expression data (cell lines). The higher the value (orange), the more relatively similar to each lineage.



#	LCDM +
1	Control
2	RA 0.1uM + FGF2
3	RA 0.1uM + ACTIVIN-A
4	RA 0.1uM + Chiron
5	RA 0.1uM + PDGF-AB
6	RA 1uM + FGF2
7	RA 1uM + ACTIVIN-A
8	RA 1uM + Chiron
9	RA 1uM + PDGF-AB
10	RA 10uM + FGF2
11	RA 10uM + ACTIVIN-A
12	RA 10uM + Chiron
13	RA 10uM + PDGF-AB
14	FGF2 + ACTIVIN-A
15	FGF2 + Chiron
16	FGF2 + PDGF-AB
17	Chiron + ACTIVIN-A
18	Chiron + PDGF-AB
19	PDGF-AB + ACTIVIN-A

#	Media
1	hEPSC
2	N2B27
3	hEPSC+10 ng/ul ACT A+2uM Chiron+hLIF
4	N2B27+10 ng/ul ACT A+2uM Chiron+hLIF
5	hEPSC+100 ng/ul ACT A+3uM Chiron+hLIF
6	N2B27+100 ng/ul ACT A+3uM Chiron+hLIF

Figure 1—figure supplement 1. 6-Day molecular screen using RUES2 human pluripotent stem cells (hPSCs). (**A**) Diagram outlining the screen procedure. At the beginning of the screen human extended pluripotent stem cells (hEPSCs) harbour a predominantly SOX2+ population. After 6 days (6D), average levels of SOX17 and SOX2 were determined. Endoderm differentiation is indicated by a concomitant increase in SOX17 and decrease in SOX2 levels. (**B**) Table of conditions used within the screen. (**C**) Bar chart depicting the average fluorescence intensity (± SEM) of SOX2 and SOX17 as measured by SOX2::mCitrine and SOX17::H2B-tdTomato+ levels via fluorescent intensity arbitrary units (a.u.). hEPSC medium (LCDM) was supplemented with pairs of pathway activators, each combination annotated with a number (n=1 independent experiment with one sample). Table in

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panel B should be used as a key for each medium condition on the x-axis. (**D**) Table of conditions used in the second screen used to optimise basal media and chemokine concentration. (**E**) Bar chart depicting the average fluorescence intensity (± SEM) of SOX2 and SOX17 as measured by SOX2::mCitrine and SOX17::H2B-tdTomato+ levels via fluorescent intensity a.u. (n=1 independent experiment with one sample). Table in panel D should be used as a key for each medium condition on the x-axis. (**F**) Line graph depicting relative increase in cell numbers of hEPSC, Rset hPSC, or human embryonic stem cell (hESC) cultured in ACTIVIN-A, Chiron, and LIF (ACL), or hEPSC, Rset hPSC, or hESC cultured in ACTIVIN-A, Chiron, and JAK inhibitor (ACi) over 6 days of conversion (± SEM). Cell numbers were calculated every 24 hr and normalised to the first day of conversion. Tukey's multiple comparisons test result for the comparison between day 6 experimental condition (ACi) and control (ACL) for hEPSC, Rset, and hESC; ****$p < 0.0001$ (n=3 independent experiments each with one sample).

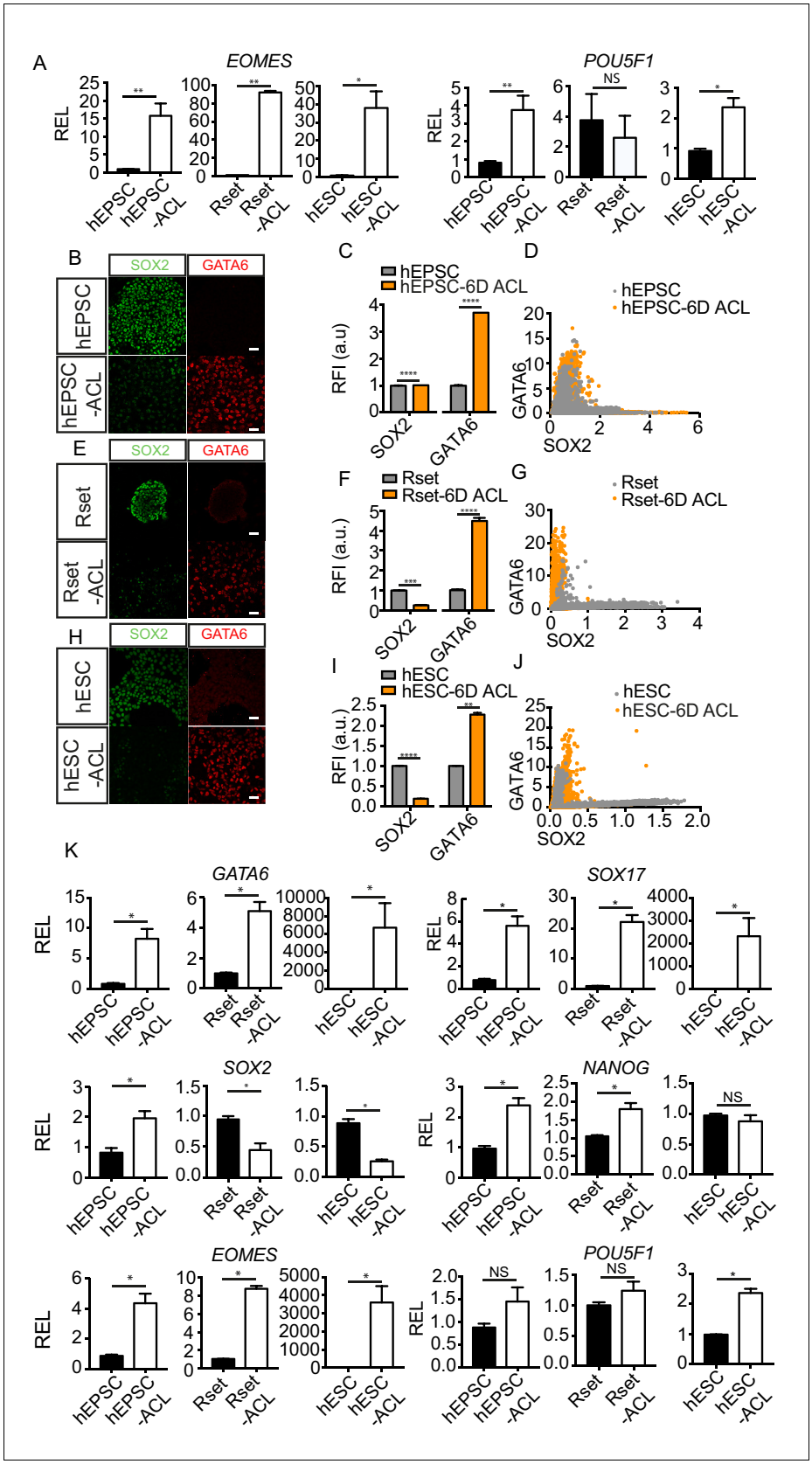

Figure 1—figure supplement 2. Endoderm conversion of RUES2 and H9 human pluripotent stem cells (hPSCs) in response to ACL treatment. (**A**) Relative expression levels (REL) (± SEM) of *EOMES* and *POU5F1* in RUES2 human

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extended pluripotent stem cell (hEPSC), Rset hPSCs (n=6 samples, three independent experiments) or hESCs (n=4 samples, two independent experiments) before and after 6 days (6D) ACL treatment, normalised to their respective control. Mann–Whitney U-test *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (**B, E, and H**) Immunofluorescence images of SOX2 (green) and GATA6 (red) in H9 hEPSCs, human embryonic stem cells (hESCs), and Rset hPSCs after 6D of ACL treatment (scale bar = 50 μm). (**C, F, and I**) Bar chart of relative fluorescence intensity (RFI) of GATA6 and SOX2 (± SEM) in H9 hEPSCs, Rset, and hESCs before and after 6D of ACL treatment as measured via protein immunostaining. Mann–Whitney U-test (number of cells analysed: hEPSC control n=2375, hEPSC + 6D ACL n=2139, Rset control n=2472, Rset + 6D ACL n=1739, hESC n=3227, hESC + 6D ACL n=3661 n = 3 independent experiments each with one sample). (**D, G, and J**) Quantification of SOX2 and GATA6 protein levels at the single cell level in H9 hEPSC, Rset hPSC, and hESC 6D ACL-treated cells measured by protein immunostaining. Each dot represents an individual cell. (**K**) REL (± SEM) of *GATA6, SOX17, SOX2, EOMES, NANOG*, and *POU5F1* in H9 hEPSC, hESCs, or Rset hPSCs after 6D ACL treatment, normalised to their respective control (n=4 samples, two independent experiments). Mann–Whitney U-test *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

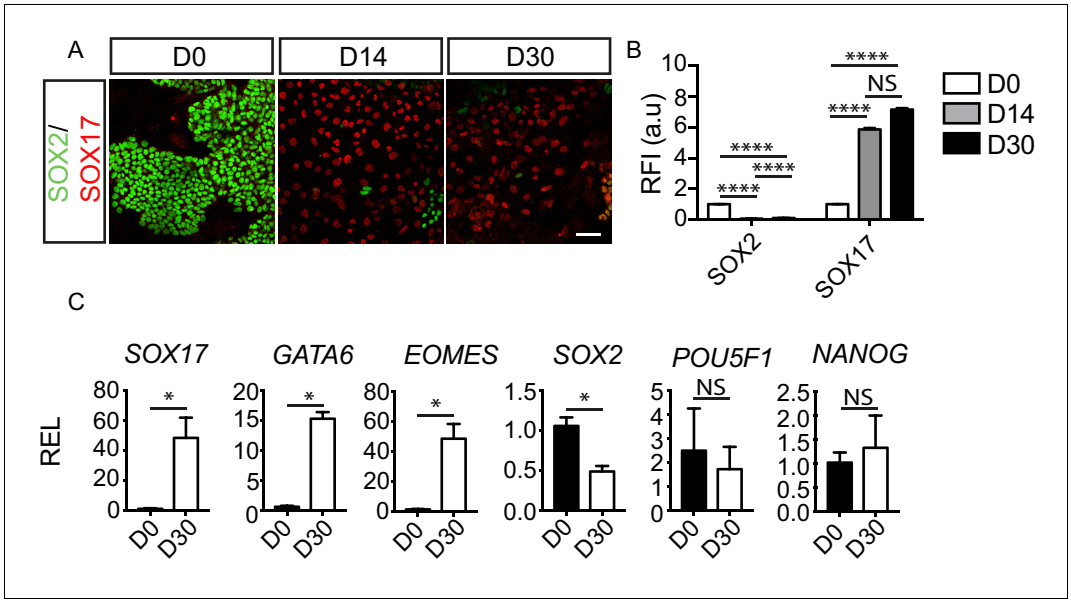

Figure 1—figure supplement 3. Prolonged culture of Rset-ACL. (**A**) Immunofluorescence images of SOX2 (green) and SOX17 (red) in RUES2 Rset human pluripotent stem cells (hPSCs) on D0, D14, and D30 of culture in ACL (scale bar = 50 μm). (**B**) Bar chart of relative fluorescence intensity (RFI arbitrary units [a.u.]) of SOX17 and SOX2 (± SEM) in Rset hPSCs on D0, D14, and D30 of culture in ACL as measured via reporter protein immunostaining. Kruskal-Wallis test with Dunn's multiple comparisons test; ****$p < 0.0001$ (number of cells analysed: D0 n=5580, D14 n=1846, D30 n=3003, three independent experiments each with one sample). (**C**) Relative expression levels (REL) (± SEM) of *SOX17, GATA6, EOMES, SOX2, POU5F1, and NANOG* (n=4 samples, two independent experiments) after 30 days of culture in ACL normalised to D0 control. Mann–Whitney U-test; NS = not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

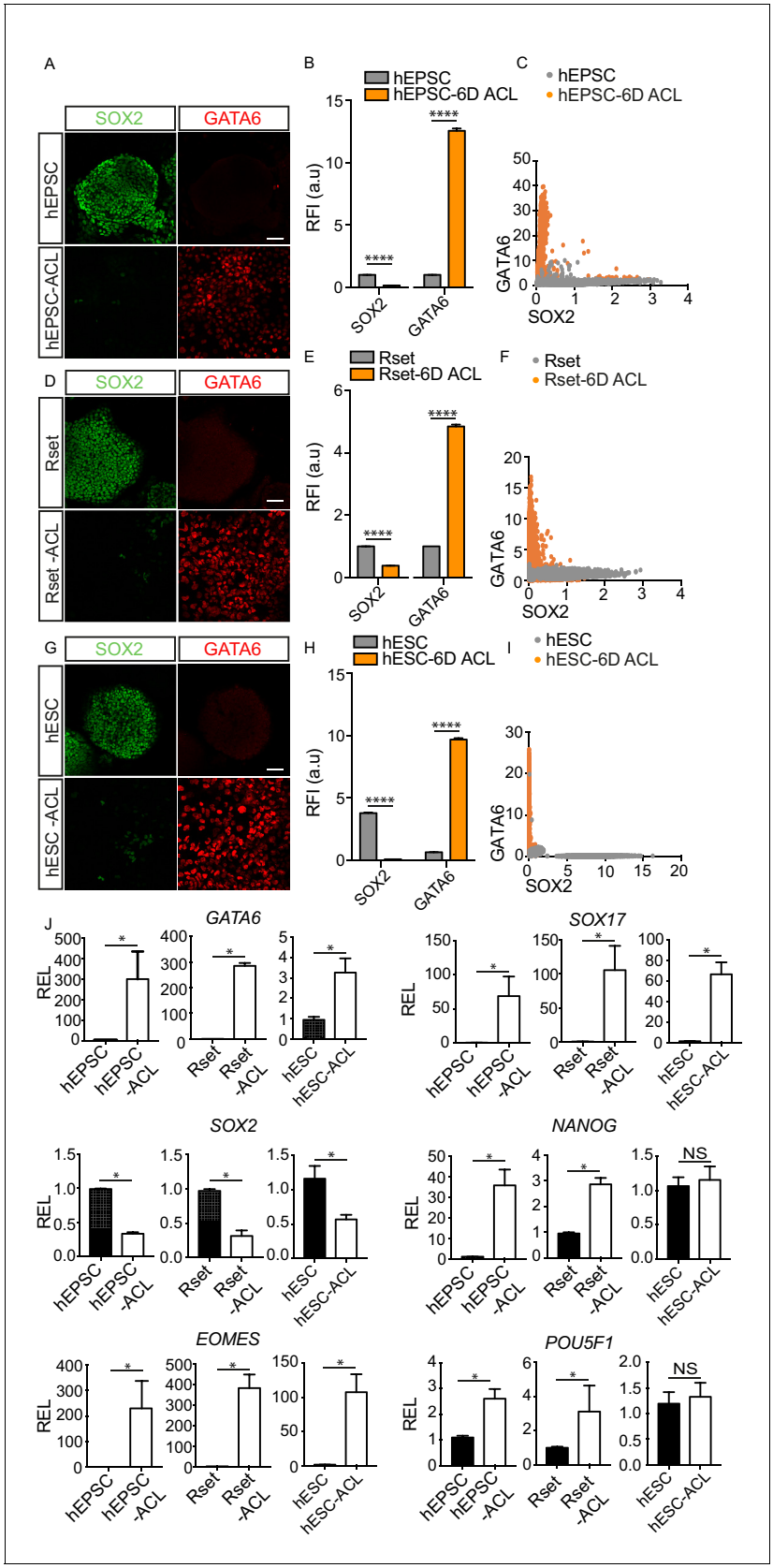

Figure 1—figure supplement 4. Endoderm conversion of SHEF6 human pluripotent stem cells (hPSCs) in response to ACL treatment. (**A, D, and G**) Immunofluorescence images of SOX2 (green) and GATA6 (red) in human extended pluripotent stem cells (hEPSCs), Rset hPSCs, and human embryonic

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stem cells (hESCs) after 6 days (6D) of ACL treatment (scale bar = 50 μm). (**B, E, and H**) Bar chart of relative fluorescence intensity (RFI arbitrary units [a.u.]) of GATA6 and SOX2 (± SEM) before and after 6D of ACL treatment in hPSCs as measured via protein immunostaining. Mann–Whitney U-test; ****p < 0.0001 (number of cells analysed: hEPSC control n=3865, hEPSC + 6D ACL n=2454, Rset control n=4125, Rset + 6D ACL n=3274, hESC control n=7020, hESC + 6D ACL n=2986, three independent experiments each with one sample). (**C, F, and I**) Quantification of SOX2 and GATA6 protein levels at the single cell level in hEPSC, Rset hPSC, and hESC 6D ACL-treated cells based on protein immunostaining. Each dot represents an individual cell. (**J**) Relative expression levels (REL) (± SEM) of *GATA6, SOX17, NANOG, SOX2, EOMES*, and *POU5F1* in hEPSCs, Rset hPSCs, and hESCs before and after 6D ACL treatment, normalised to their respective control (n=4 samples, two independent experiments) Mann–Whitney U-test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

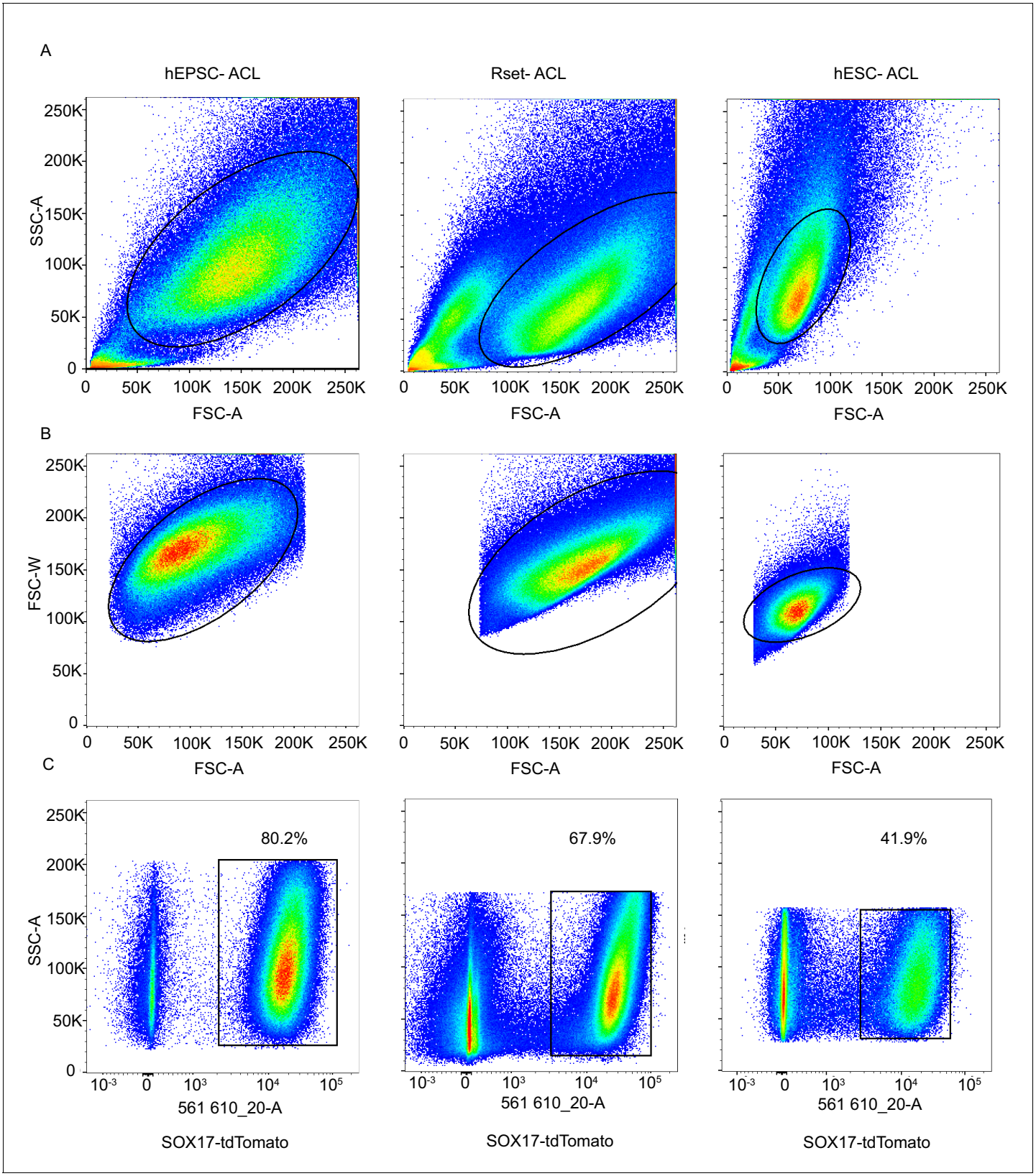

Figure 1—figure supplement 5. Flow cytometry gating strategy for isolating the SOX17::tdTomato+ cells within human extended pluripotent stem cell (hEPSC)-ACL, Rset-ACL, and human embryonic stem cell (hESC)-ACL populations. (**A**) Gating strategy for separating debris from cell population. (**B**) Gating strategy for isolating single cells from doublets. (**C**) Gating strategy to separate SOX17::tdTomato+ from SOX17::tdTomato- population of hEPSC-ACL and Rset-ACL and hESC-ACL populations. The percentage of the population which was SOX17::tdTomato+ is shown on the graphs.

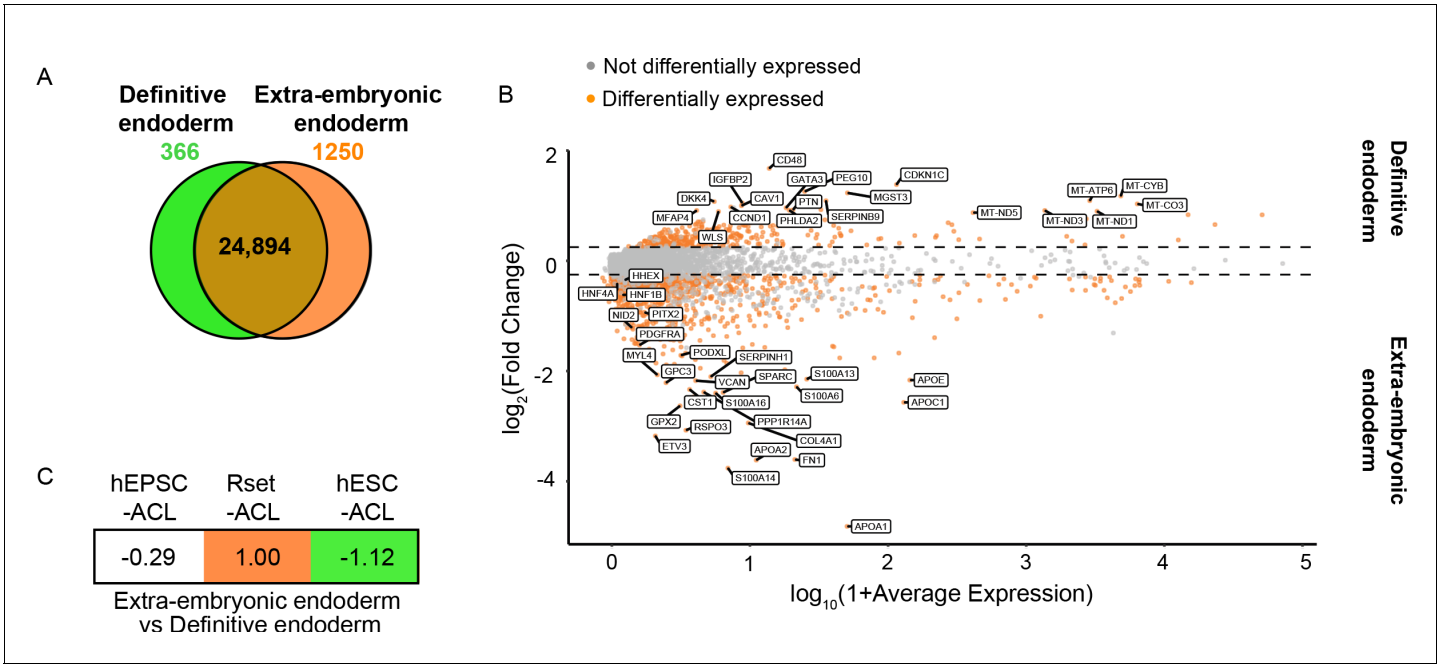

Figure 2. Transcriptional profiling of ACL-treated human pluripotent stem cells (hPSCs) relative to extra-embryonic endoderm and definitive endoderm expression signatures. (**A**) Venn diagram depicting the number of shared genes between human extra-embryonic endoderm (E6–14) and human embryonic stem cell (hESC)-derived definitive endoderm (brown), of extra-embryonic endoderm differentially expressed genes (orange), and of definitive endoderm differentially expressed genes (green). (**B**) MA plot representing the differentially expressed genes between human definitive endoderm and extra-embryonic endoderm. Top 20 differentially expressed genes are labelled, along with key extra-embryonic endoderm markers NID2, PDGFRa, HNF4a, HNF1β, PITX2, PODXL, and HHEX (>10% of cell type of interest, $log_2FC>0.25$, $p<0.05$). (**C**) Transcriptomic-signature comparison score of human extra-embryonic endoderm vs. definitive endoderm within ACL-treated hPSCs (gene set variation analysis ([GSVA]) score for negative markers subtracted from GVSA score for positive markers and values normalised to 1). A negative value represents definitive endoderm similarity and a positive value represents an extra-embryonic endoderm similarity. hEPSC-ACL: n=2 technical replicates, Rset-ACL: n=3 technical replicates, hESC-ACL: n=2 technical replicates. Sc-RNA-seq expression data (extra-embryonic endoderm and definitive endoderm) and bulk RNA-seq expression data (cell lines) were used for all relevant panels.

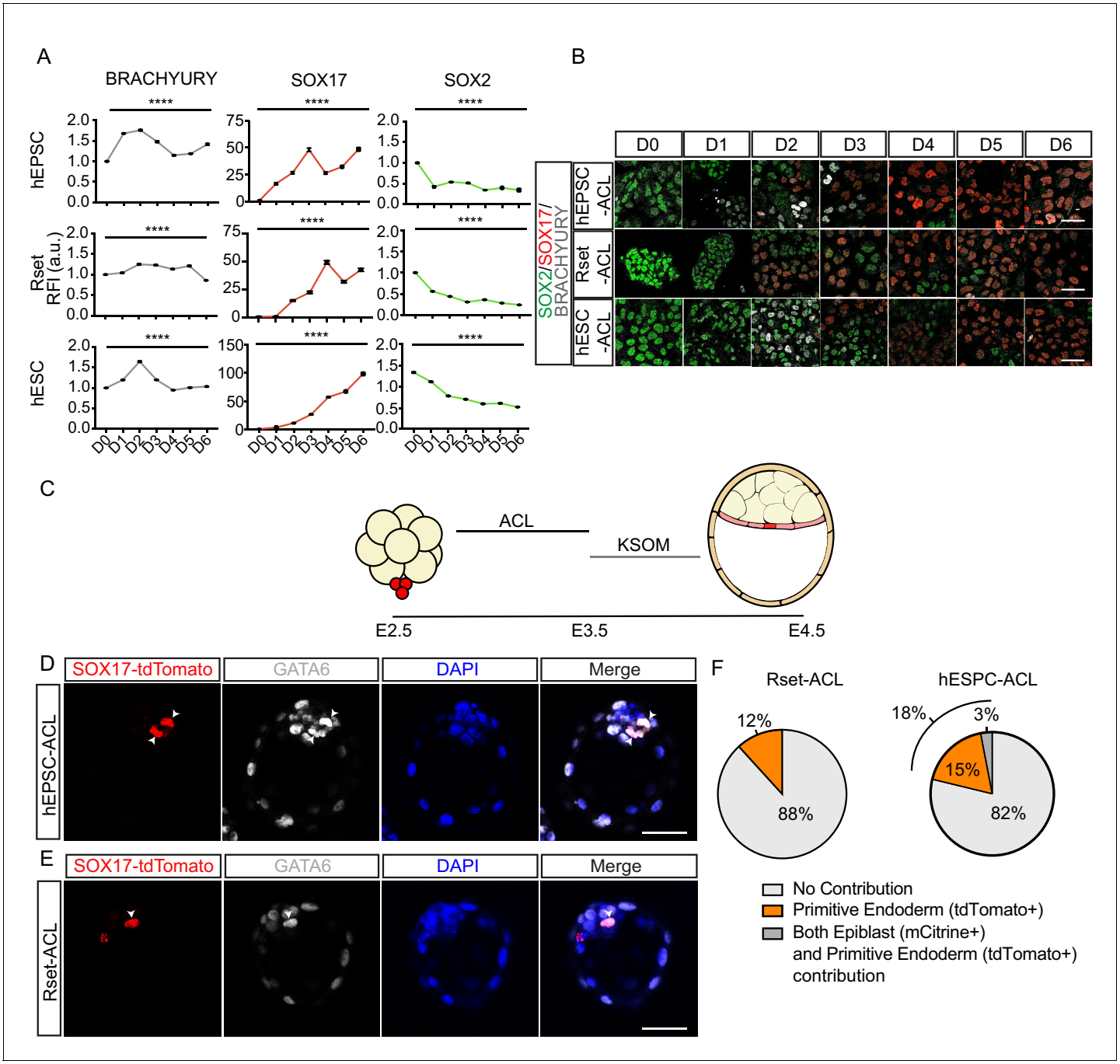

Figure 3. Functional characterisation of ACL-treated human pluripotent stem cells (hPSCs). (**A**) Line graph (± SEM) plotting the relative fluorescence intensity (RFI arbitrary units [a.u.]) of SOX2, SOX17, and BRACHYURY in ACL-treated population. Kruskal–Wallis test, ****p < 0.0001 (number of cells analysed SOX17: human extended pluripotent stem cell (hEPSC): D0 n=2774, D1 n=3258, D2 n = 2932, D3 n=3292, D4 n=2459, D5 n=2293, D6 n=1883, Rset: D0 n=2108, D1 n=3580, D2 n=2738, D3 n=2586, D4 n=2149, D5 n=2698, D6 n = 2441, human embryonic stem cell (hESC): D0 n=1426, D1 n=1924, D2 n=1943, D3 n=2817, D4 n=2248, D5 n=1148, D6 n=1957 (n=2 independent experiments); SOX2: hEPSC: D0 n = 2774, D1 n=3258, D2 n=2932, D3 n=3292, D4 n=2459, D5 n=2293, D6 n=1883, Rset: D0 n=2342, D1 n=3634, D2 n=2794, D3 n=2671, D4 n=2068, D5 n=2293, D6 n=2918, hESC: D0 n=2353, D1 n=3082, D2 n=3129, D3 n=4174, D4 n=2989, D5 n=1787, D6 n=3114; BRACHYURY: hEPSC: D0 n = 3452, D1 n=4090, D2 n=3435, D3 n=4223, D4 n=2881, D5 n=2837, D6 n=2218, Rset: D0 n=2317, D1 n = 3739, D2 n=3141, D3 n=2881, D4 n=2467, D5 n=2951, D6 n=4400, hESC: D0 n=2685, D1 n=3433, D2 n=3997, D3 n=5325, D4 n=3490, D5 n=2155, D6 n = 3258. n = 3 independent experiments). (**B**) Immunofluorescence images of SOX2 (green), SOX17 (red), and BRACHYURY (grey) in hEPSCs, Rset hPSCs, and hESCs during 6 days of ACL treatment (scale bar = 50 μm). (**C**) Schematic of human-mouse chimera protocol using ACL-treated cells. Clumps of three to six SOX-17::H2B-tdTomato+ treated cells were aggregated with E2.5 mouse embryos and cultured for 24 hr in ACL. After this, embryos were moved into KSOM and cultured for another 24 hr. (**D**)

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Immunofluorescence images of SOX17::H2B-tdTomato (red), GATA6 (grey), and DAPI (blue) in E4.5 mouse blastocysts. hEPSC-ACL cells contribution denoted by SOX17::H2B-tdTomato+ (red) cells (white arrowheads) (scale bar = 50 μm). (**E**) Immunofluorescence images of SOX17::H2B-tdTomato (red), GATA6 (grey), and DAPI (blue) in E4.5 mouse blastocysts. Rset-ACL cells contribution denoted by SOX17::H2B-tdTomato+ (red) cells (white arrowheads). (**F**) Pie charts showing percentage of human cell contribution to the primitive endoderm, epiblast, or both. Rset (N = 67 embryos, n=3 independent experiments), hEPSC (N = 39 embryos, n=3 independent experiments).

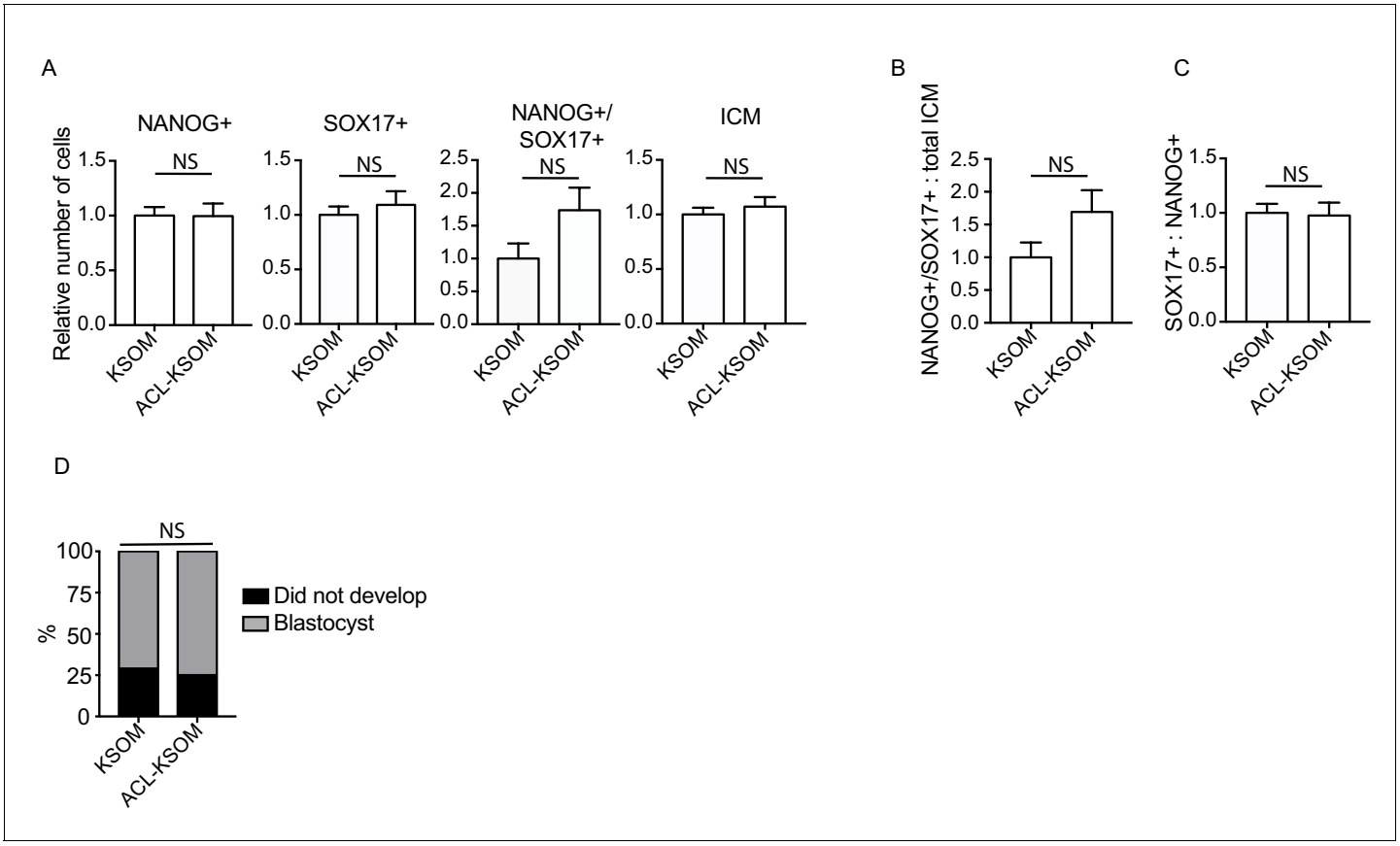

Figure 3—figure supplement 1. Characterisation of mouse blastocysts cultured in interspecies chimera conditions. (**A**) Characterisation of lineage allocation in E4.5 mouse blastocysts cultured in either KSOM or ACL-KSOM. Bar chart (± SEM) depicting the relative number of cells that were NANOG +, SOX17+, NANOG+/SOX17+, and the relative number of total cells within the inner cell mass (ICM). Unpaired Student's t-test, NS = p>0.05. (**B**) Bar chart depicting the proportion of the total ICM that consisted of NANOG+/SOX17+ cells (± SEM). Unpaired Student's t-test, NS = p>0.05. (**C**) Bar chart depicting the ratio between SOX17+ cells and NANOG+ cells within the ICM (± SEM). Unpaired Student's t-test, NS = p>0.05. For A-C: KSOM: n = 16 embryos, ACL-KSOM: n = 14 embryos, n=2 independent experiment. (**D**) Viability of E4.5 blastocysts within either KSOM (n = 14 embryos) or ACL-KSOM (n = 28 embryos) (n=1 independent experiment). Chi-squared test. NS p > 0.05.

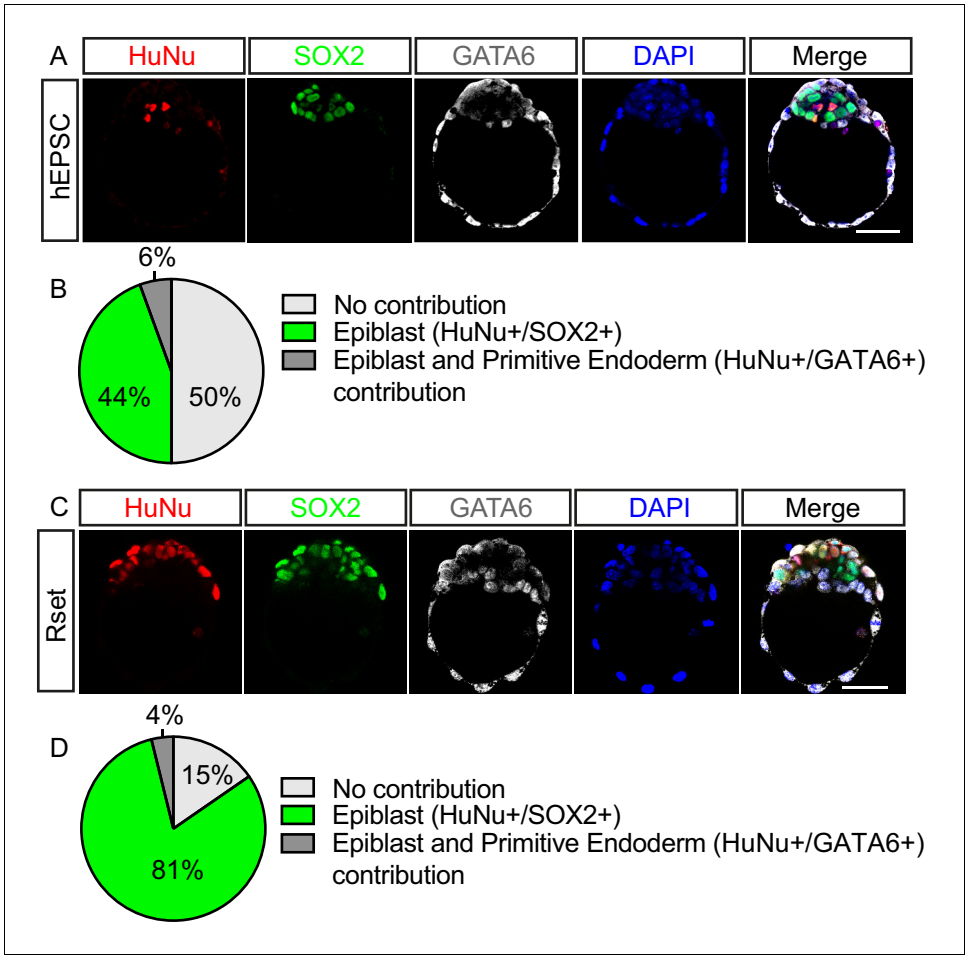

Figure 3—figure supplement 2. Contribution of human extended pluripotent stem cell (hEPSC) and Rset human pluripotent stem cells (hPSCs) to interspecies chimeras. (**A**) Immunofluorescence images of human nuclear antigen (HuNu) (red), SOX2 (green), GATA6 (grey) and DAPI (blue) in E4.5 human-mouse chimeric blastocysts demonstrating contribution of hEPSCs (scale bar = 50 μm). (**B**) Pie charts showing percentage of hEPSC contribution to the primitive endoderm, epiblast, or both (N=18 embryos, n=2 independent experiments). (**C**) Immunofluorescence images of HuNu (red), SOX2 (green), GATA6 (grey) and DAPI (blue) in E4.5 human-mouse chimeric blastocysts demonstrating contribution of Rset hPSCs (scale bar = 50 μm). (**D**) Pie charts showing percentage of Rset hPSC contribution to the primitive endoderm, epiblast, or both (N=26 embryos, n=2 independent experiments).

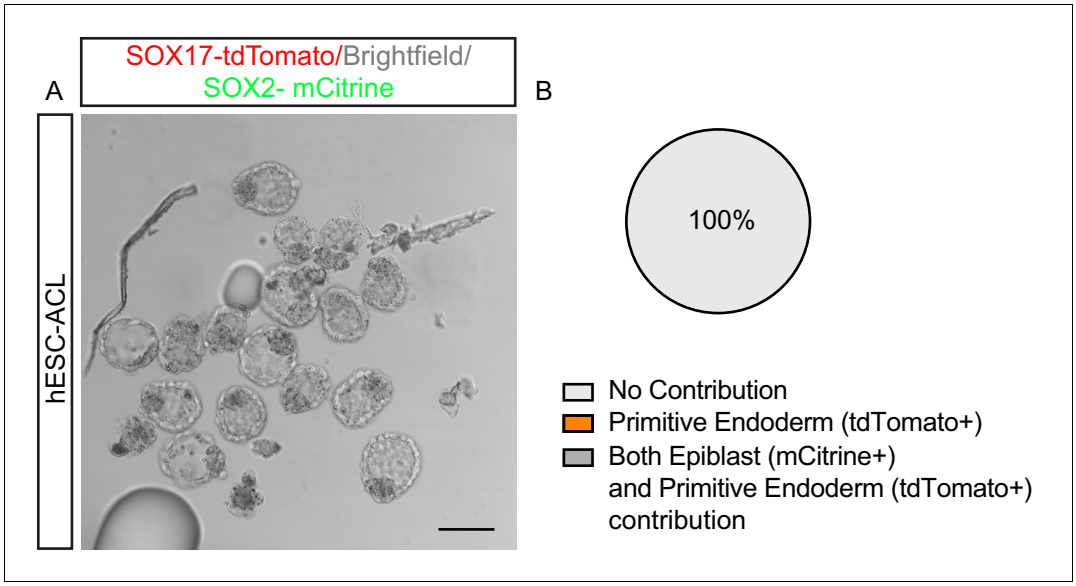

Figure 3—figure supplement 3. Contribution of ACL-treated human embryonic stem cells (hESCs) to interspecies chimeras. (**A**) Brightfield image of E4.5 mouse blastocysts after aggregation with SOX17::H2B-tdTomato+ ACL-treated hESCs (scale bar = 100 μm). (**B**) Pie charts showing percentage contribution to the primitive endoderm, epiblast, or both by SOX17::H2B-tdTomato+ ACL-treated hESCs (n = 48 embryos, n=3 independent experiments).

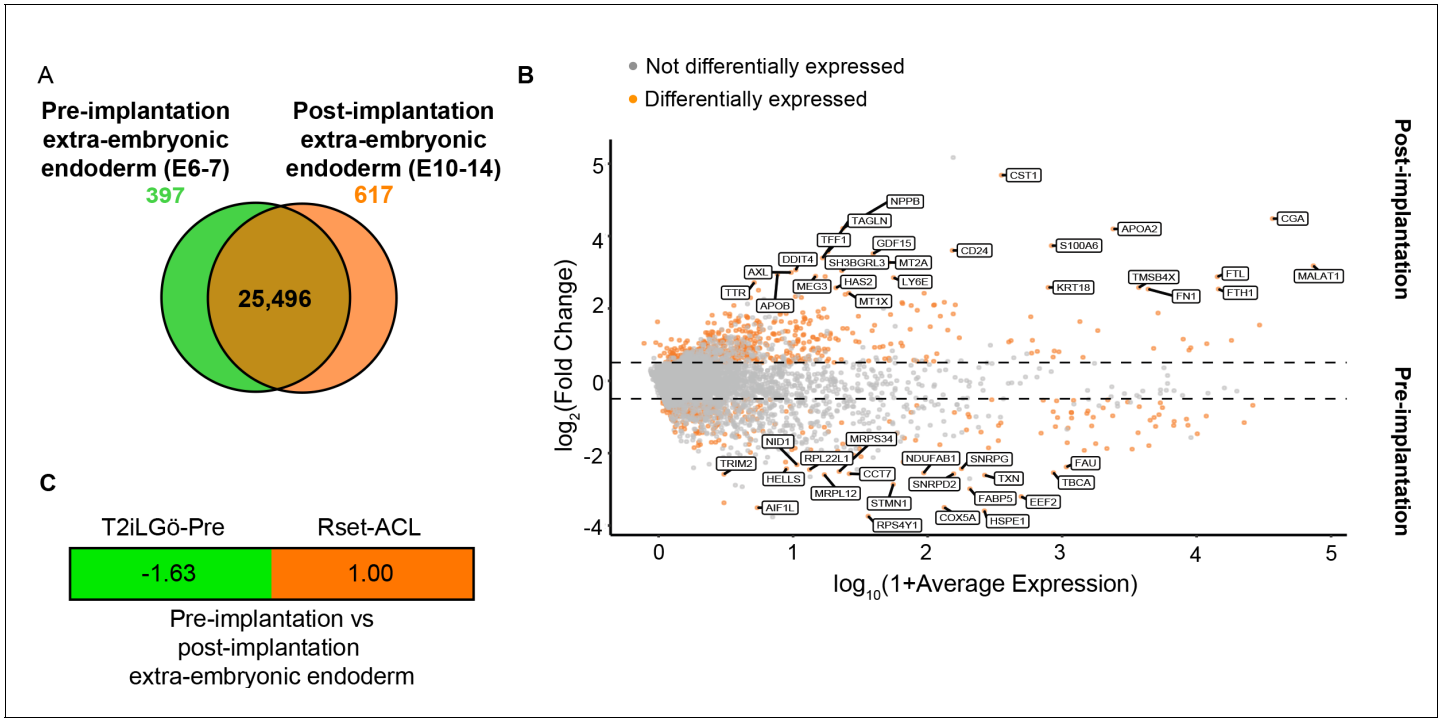

Figure 4. Transcriptional profiling of T2iLGö-PrE and Rset-ACL relative to human pre- and post-implantation extra-embryonic endoderm expression signatures. (**A**) Venn diagram depicting the number of shared genes between pre-implantation extra-embryonic endoderm (E6–7) and post-implantation extra-embryonic endoderm (E10–14) (brown), of pre-implantation extra-embryonic endoderm differentially expressed genes (green) and of post-implantation extra-embryonic endoderm differentially expressed genes (orange). (**B**) MA plot representing the differentially expressed genes between human pre-implantation and post-implantation extra-embryonic endoderm. Top 20 differentially expressed genes are labelled (>10% of cell type of interest, $log_2FC>0.25$, $p<0.05$). (**C**) Transcriptomic-signature comparison score of pre-implantation extra-embryonic endoderm vs. post-implantation extra-embryonic endoderm for T2iLGö-Pre and Rset-ACL (gene set variation analysis [GSVA] score for pre-implantation extra-embryonic endoderm subtracted from GSVA score for post-implantation extra-embryonic endoderm and values normalised to 1). A negative value (green) represents pre-implantation extra-embryonic endoderm similarity and a positive value (orange) represents post-implantation extra-embryonic endoderm. T2iLGö-Pre: n=2 technical replicates (from published dataset), Rset-ACL: n=3 technical replicates. Sc-RNA-seq expression data (pre- and post-implantation extra-embryonic endoderm) and bulk RNA-seq expression data (cell lines) were used for all relevant panels.

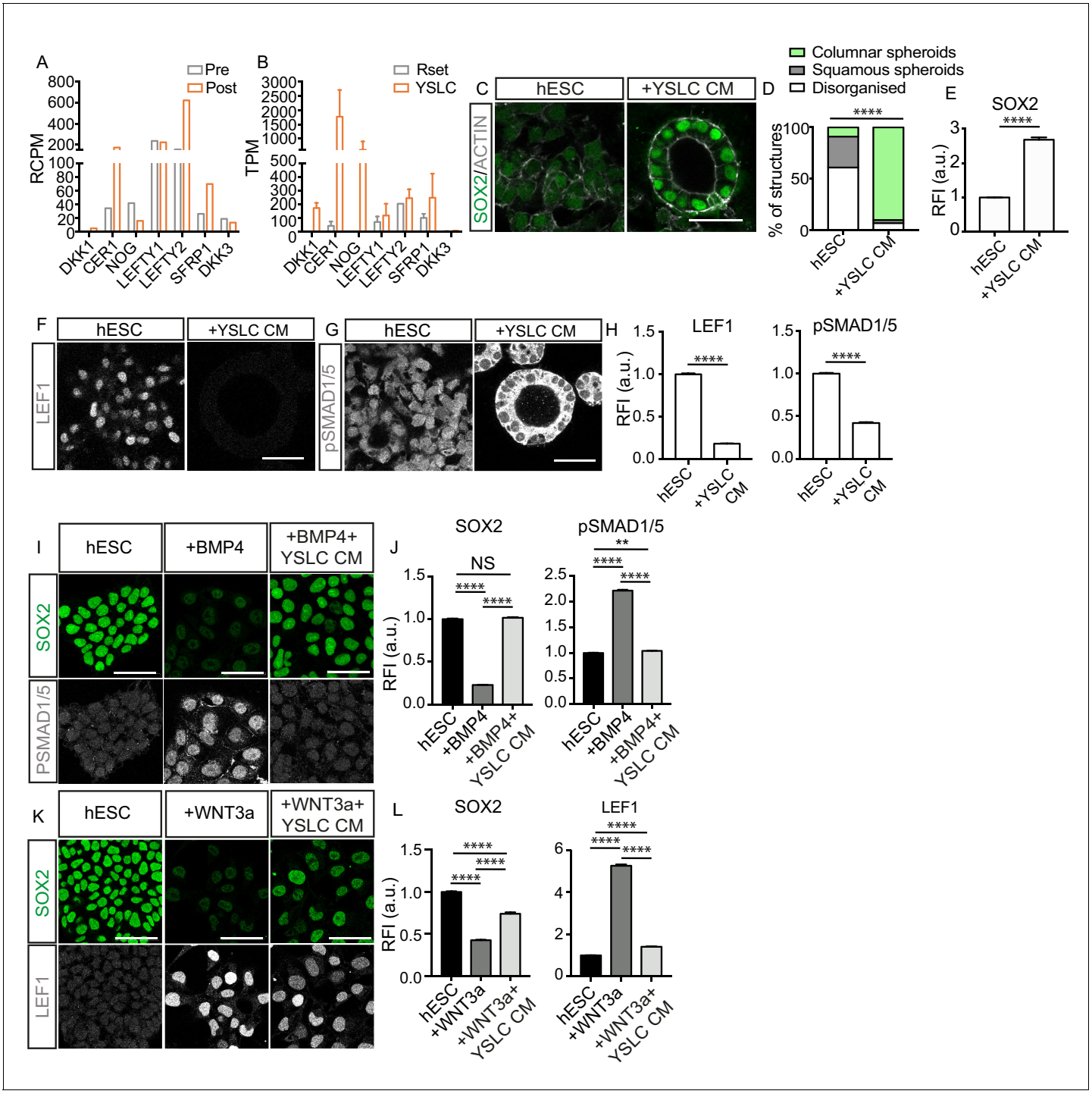

Figure 5. Yolk sac-like cells (YSLCs) antagonise BMP and WNT signalling in human embryonic stem cells (hESCs). (**A**) Bar chart plotting average expression levels of WNT, NODAL, and BMP inhibitors in pre-implantation (Pre, E6–7) and post-implantation human hypoblast (Post, E10–14) denoted as relative counts per million (RCPM) using published sc-RNA-seq data. (**B**) Bar chart (± SEM) plotting expression levels of BMP, NODAL, and WNT inhibitors in Rset hPSCs and YSLCs denoted as transcripts per million (TPM) using bulk RNA-seq data (YSLCs: n=3 technical replicates, Rset: n=2 technical replicates [from published dataset]). (**C**) Immunofluorescence images of SOX2 (green) and ACTIN (grey) in hESCs cultured in 3D Geltrex in either mTeSR (hESC) or mTeSR conditioned on YSLCs (+YSLC CM) (scale bar = 50 μm). (**D**) Percentage of structures that are columnar, squamous, or disorganised based on cell aspect ratio calculations in cells from panel C using ACTIN stainings. Chi-squared test. ****p < 0.0001 (number of structures analysed: control n=33, +YSLC CM n=30, n=3 independent experiments each with one sample). (**E**) Bar chart (± SEM) depicting SOX2 levels as inferred from relative fluorescence intensity (RFI arbitrary units [a.u.]) in cells from panel C. Mann–Whitney U-test; ****p < 0.0001 (number of cells analysed:

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SOX2: hESC n=2346, +YSLC CM n=596, n=3 independent experiments each with one sample). (**F**) Immunofluorescence of pSMAD1/5 (grey) in hESCs cultured in mTeSR (hESC) or mTeSR conditioned on YSLCs (+YSLC CM) in 3D Geltrex (scale bar = 50 μm). (**G**) Immunofluorescence of LEF1 (grey) in hESCs cultured in mTeSR (hESC) or mTeSR conditioned on YSLCs (+YSLC CM) in 3D Geltrex (scale bar = 50 μm). (**H**) Bar chart (± SEM) depicting the levels of pSMAD1/5 (nuclear) and LEF1 in hESCs cultured in mTeSR (hESC) or mTeSR conditioned on YSLCs (+YSLC CM) as inferred from RFI in cells from panels F and G, respectively. Mann–Whitney U-test, ****p <0.0001 (number of cells analysed: pSMAD1/5: hESC n=2563, +YSLC CM n=1660; LEF1: hESC n=1984, +YSLC CM n=1041. n=3 independent experiments each with one sample). (**I**) Immunofluorescence of SOX2 (green) and pSMAD1/5 (grey) in hESCs cultured in mTeSR (hESC), treated with BMP4 (+BMP4) or treated with BMP4 and YSLC conditioned media (+BMP4+YSLC CM) (scale bar = 50 μm). (**J**) Bar charts (± SEM) depicting the levels of SOX2 and pSMAD1/5 RFI in samples from panel I. Kruskal–Wallis test with Dunn's multiple comparisons test, ****p<0.0001 (number of cells analysed: hESC n=1549, +BMP4 n = 1063, +BMP4+YSLC CM n=1544, n=3 independent experiments each with one sample). (**K**) Immunofluorescence of SOX2 (green) and LEF1 (grey) in hESCs cultured in mTeSR (hESC), treated with WNT3a (+WNT3a) or treated with WNT3a and YSLC conditioned media (+WNT3a+YSLC CM) (scale bar = 50 μm). (**L**) Bar chart (± SEM) depicting the levels of SOX2 and LEF1 RFI in samples from panel K. Kruskal–Wallis test with Dunn's multiple comparisons test, ****p<0.0001 (number of cells analysed: hESC n=1604, +WNT3a n=1682, +WNT3a+YSLC CM n=1376, n=3 independent experiments each with one sample).

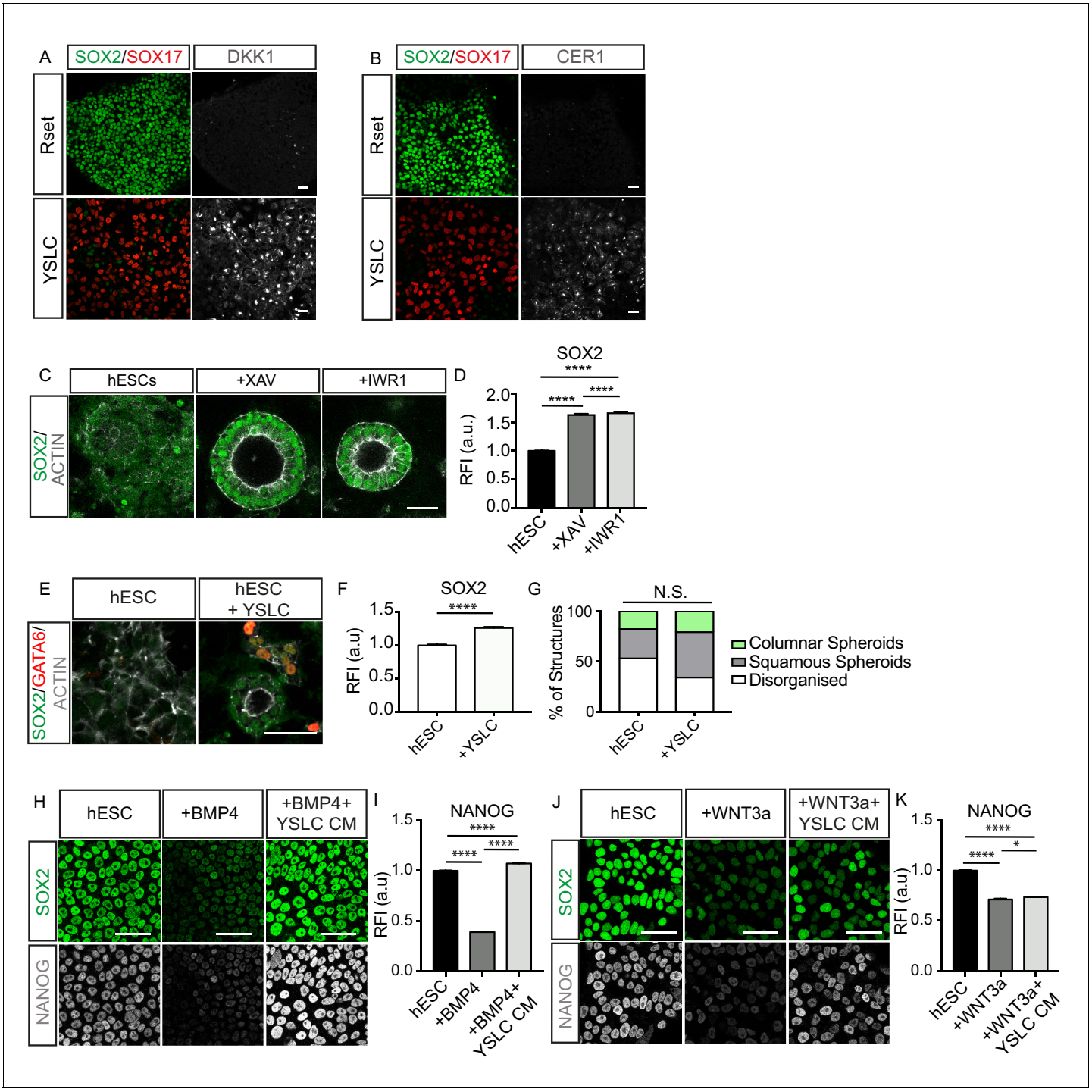

Figure 5—figure supplement 1. Yolk sac-like cells (YSLCs) prevent SOX2 and NANOG loss. (**A**) Immunofluorescence of SOX2 (green), SOX17 (red), and DKK1 (grey) in Rset and YSLCs (after 6D ACL treatment) (scale bar = 50 μm). (**B**) Immunofluorescence of SOX2 (green), SOX17 (red), and CER1 (grey) in Rset and YSLCs (after 6D ACL treatment) (scale bar = 50 μm) (**C**) Immunofluorescence of SOX2 (green) and ACTIN (grey) within human embryonic stem cells (hESCs) cultured in 3D Geltrex for 72 hr (scale bar = 50 μm). (**D**) Bar chart (± SEM) depicting relative fluorescence intensity (RFI arbitrary units [a.u]) of SOX2 as studied via protein immunostaining in hESCs cultured in 3D Geltrex alone or in the presence of the WNT inhibitors XAV (+XAV) or IWR1 (+IWR1). Kruskal–Wallis test with Dunn's multiple comparisons test. NS: $p > 0.05$, ****$p < 0.0001$ (number of cells analysed: control n=3273, +XAV n=2220, +IWR1 n=2234, n = 3 independent experiments each with one sample). (**E**) Immunofluorescence images of SOX2 (green), GATA6 (red), and ACTIN (grey) in hESCs and hESC-YSLC co-cultures after 72 hr in 3D Geltrex (scale bar = 50 μm). (**F**) Bar chart (± SEM) depicting SOX2 levels as calculated using relative fluorescence intensity (RFI a.u.) in cells from panel E. Mann–Whitney U-test. ****$p < 0.0001$ (number of cells analysed: control n=773, hESC+YSLC=1198, n=3 independent experiments each with one sample). (**G**) Percentage of structures that are columnar, squamous, or

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disorganised based on cell aspect ratio calculations in cells from panel E using ACTIN stainings. Chi-squared test; NS p >0.05 (number of structures analysed: hESC n = 34, hESC+YSLC n=28. n=3 independent experiments each with one sample). (**H**) Immunofluorescence of SOX2 (green) and NANOG (grey) in hESCs, hESCs treated with BMP4 (+BMP4) or treated with BMP4 and YSLC conditioned media (CM) (+BMP4+YSLC CM) (scale bar = 50 μm). (**I**) Bar charts (± SEM) depicting the levels of NANOG RFI in samples from panel H. Kruskal–Wallis test with Dunn's multiple comparisons test, ****p<0.0001 (number of cells analysed: hESC n=6736, +BMP4 n=8659, +BMP4+YSLC CM n=7255, n=3 independent experiments each with one sample). (**J**) Immunofluorescence of SOX2 (green) and NANOG (grey) in hESCs, hESCs treated with WNT3a (+WNT3a) or treated with WNT3a and YSLC CM (+WNT3a+YSLC CM) (scale bar = 50 μm). (**K**) Bar chart (± SEM) depicting the levels of NANOG RFI in samples from panel J. Kruskal–Wallis test with Dunn's multiple comparisons test. ****p<0.0001, *p < 0.05 (number of cells analysed: hESC n=3225, +WNT3a n=2702, +WNT3a+YSLC CM n=3723, n=3 independent experiments each with one sample).

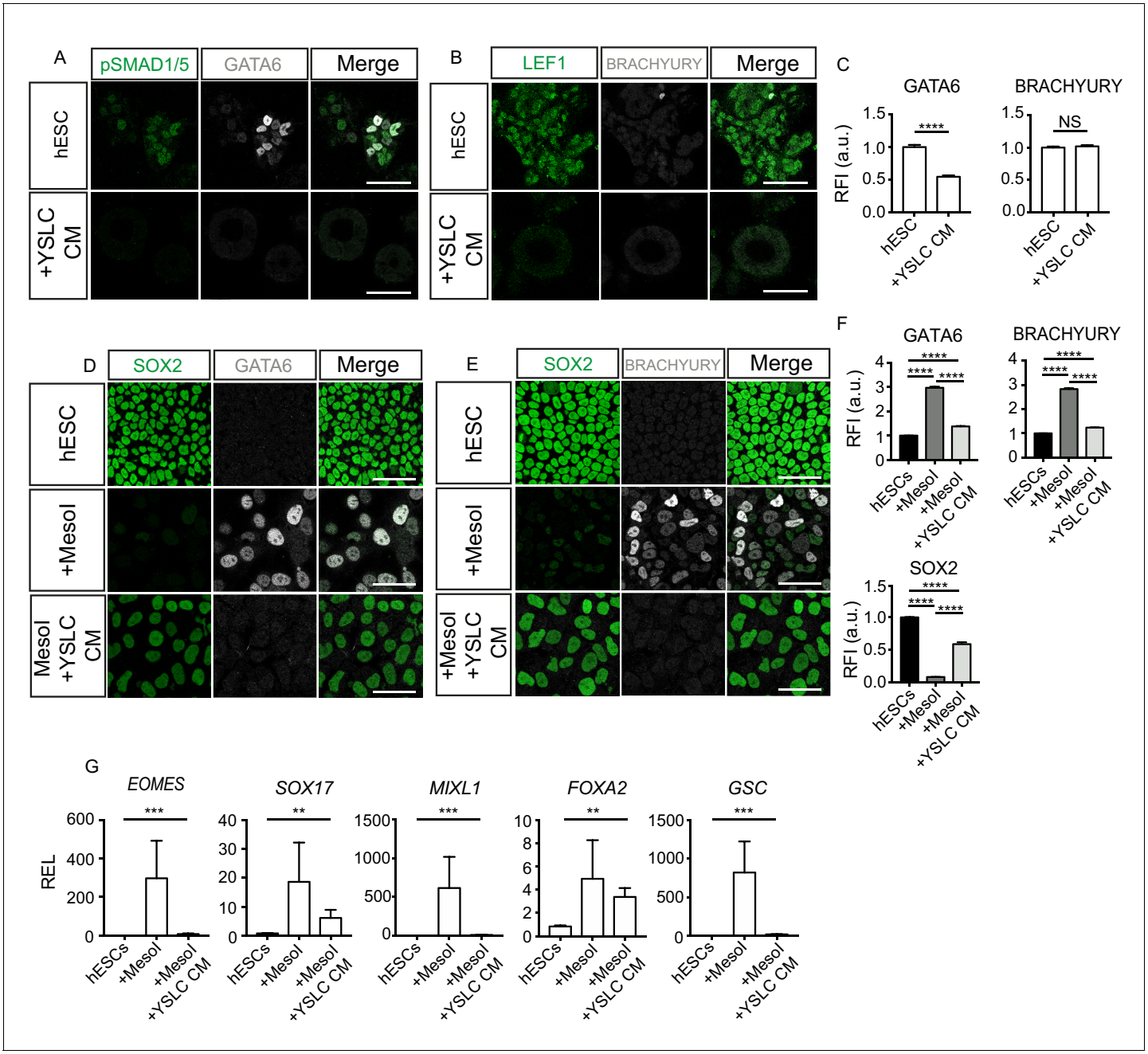

Figure 6. Yolk sac-like cells (YSLCs) inhibit mesoderm and endoderm specification in human embryonic stem cells (hESCs) in 3D and 2D. (**A**) Immunofluorescence of pSMAD1/5 (green) and GATA6 (grey), and a merge of the two, in hESCs cultured in 3D Geltrex in mTeSR (hESC) or YSLC conditioned media (+YSLC CM) (scale bar = 50 μm). (**B**) Immunofluorescence of LEF1 (green) and BRACHYURY (grey), and a merge of the two, in hESCs cultured in 3D Geltrex in mTeSR (hESC) or YSLC conditioned media (+YSLC CM) (scale bar = 50 μm). (**C**) Bar charts (± SEM) depicting the levels of GATA6 and BRACHYURY in hESCs cultured in 3D Geltrex in mTeSR (hESC) or YSLC conditioned media (+YSLC CM) as inferred from relative fluorescence intensity (RFI) arbitrary units [a.u.] in cells from panels A and B, respectively (number of cells analysed: GATA6: hESC n=2336, YSLC CM n=1074, BRACHURY: hESC n=2112, YSLC CM n=1261, n=3 independent experiments each with one sample) Mann–Whitney U-test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (**D**) Immunofluorescence of SOX2 (green) and GATA6 (grey), and a merge of the two in hESCs, hESCs in Mesol (mesoderm induction medium), or Mesol + YSLC CM (scale bar = 50 μm). (**E**) Immunofluorescence of SOX2 (green) and BRACHYURY (grey), and a merge of the two, in hESCs, hESCs in mesoderm induction medium (Mesol), or Mesol conditioned on YSLCs (Mesol + YSLC CM) (scale bar = 50 μm). (**F**) Bar chart (± SEM) depicting the levels of GATA6, BRACHYURY and SOX2 in hESCs cultured in basal medium (hESC), hESCs in mesoderm induction medium (Mesol), or Mesol + YSLC CM as measured by RFI. Kruskal–Wallis test with Dunn's multiple comparisons test. ****p <0.0001 (number of cells analysed: GATA6: hESC n = 5583, +Mesol n=2554, +Mesol+YSLC CM n=2383, BRACHYURY: hESC n = 6859, +Mesol n=6741, +Mesol+YSLC CM n=2072; SOX2: hESC n=6859, +Mesol n=6741, +Mesol+ YSLC CM n=2072. n=3 independent experiments each with one sample). (**G**) Relative

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expression levels (REL) (± SEM) of *EOMES*, *SOX17*, *MIXL1*, *FOXA2*, and *GSC* in hESCs cultured in basal medium (hESC), hESCs in mesoderm induction medium (MesoI), or MesoI + YSLC CM as measured by RFI, normalised to their hESC control (n=4 samples, two independent experiments). Kruskal–Wallis test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

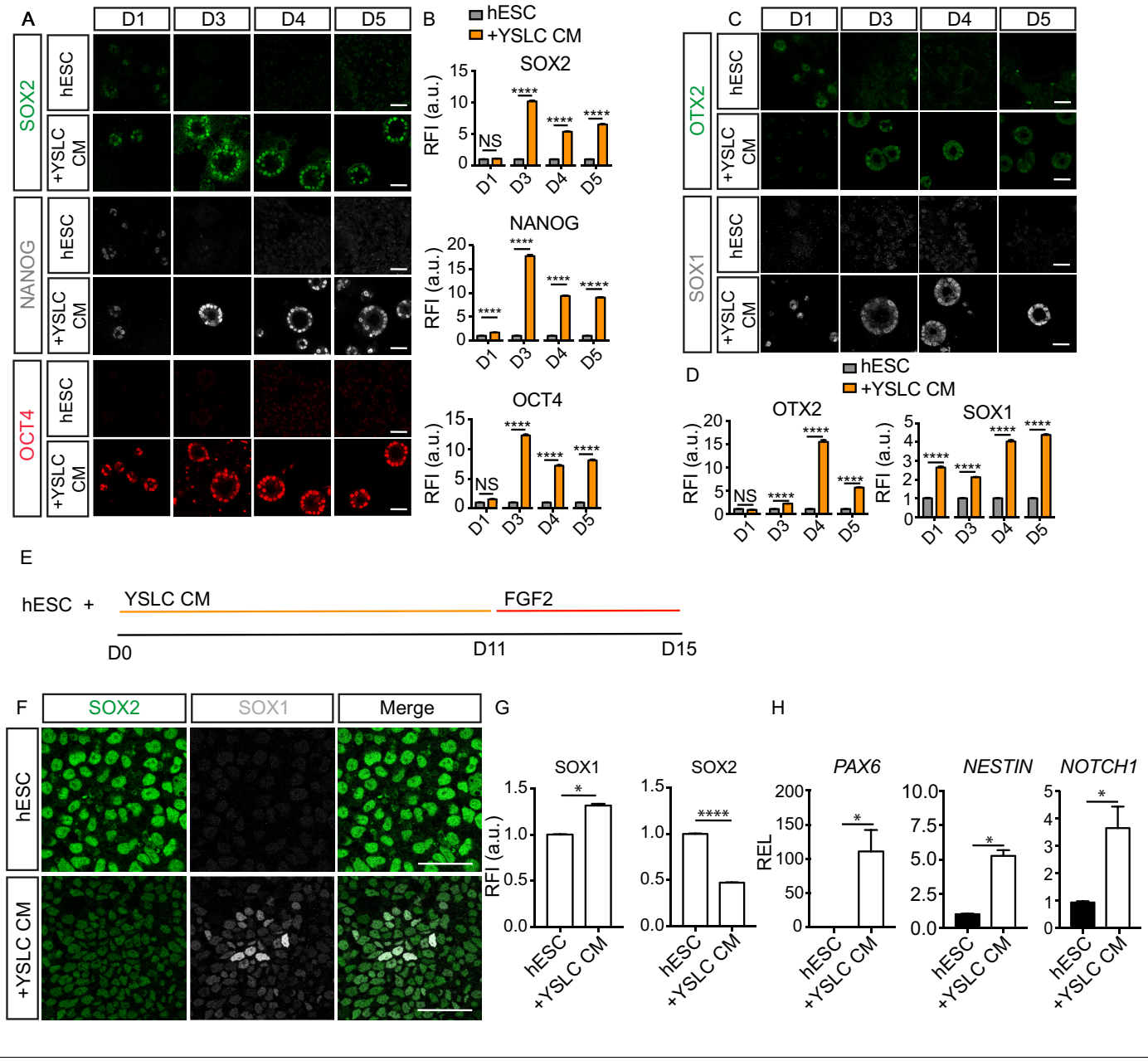

Figure 7. Yolk sac-like cells (YSLCs) induce the co-expression of anterior ectoderm markers and pluripotency markers in human embryonic stem cell (hESC) in 3D and 2D. (**A**) Immunofluorescence of SOX2 (green), NANOG (grey), and OCT4 (red) in hESCs after 1, 3, 4, or 5 days cultured in mTeSR (hESC) or in YSLC conditioned media (+YSLC CM) in 3D Geltrex (scale bar = 50 μm). (**B**) Bar charts (± SEM) depicting relative fluorescence intensity (RFI arbitrary units [a.u.]) of SOX2, NANOG, and OCT4 in hESCs cultured in YSLC CM (+YSLC CM) relative to hESCs cultured in mTeSR (hESCs) in 3D Geltrex for 1, 3, 4, and 5 days. Two-way ANOVA with Sidak's multiple comparisons test comparing hESC vs. +YSLC CM for each day. ****p<0.0001, ** <0.01, NS p>0.05 (number of cells analysed: SOX2: day 1: hESC n=567, +YSLC CM n=662, day 3: hESC n=2318, +YSLC CM n=1870, day 4: hESC n=3755, +YSLC CM n=1249, day 5: hESC n=4946, +YSLC CM n=1897, OCT4: day 1: hESC n=450, +YSLC CM n=601, day 3: hESC=1929, +YSLC CM n=1902, day 4: hESC=3472, +YSLC CM n=1314, day 5: hESC n=4946, +YSLC CM n=1897, NANOG: day 1: hESC n=450, +YSLC CM n=601, day 3: hESC n=1929, +YSLC CM n=1902, day 4: hESC n=3472, +YSLC CM n=1314, day 5: hESC n=4946, +YSLC CM n=1897, n = 3 independent experiments each with one sample). (**C**) Immunofluorescence of OTX2 (green) and SOX1 (grey) in hESCs after 1, 3, 4, or 5 days cultured in mTeSR (hESC) or YSLC conditioned media (+YSLC CM) in 3D Geltrex (scale bar = 50 μm). (**D**) Bar charts (± SEM) depicting RFI of OTX2 and SOX1 in hESCs cultured in YSLC CM (+YSLC CM) relative to hESCs cultured in mTeSR (hESC) in 3D Geltrex for 1, 3, 4, and 5 days. Two-way ANOVA with Sidak's multiple comparisons test comparing hESC vs. +YSLC CM for each day. ****p<0.0001 NS, p>0.05 (number of cells analysed: OTX2: day 1: hESC n=456, YSLC CM n=914, day

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3: hESC n=4278, +YSLC CM n=2036, day 4: hESC n=2432, +YSLC CM n=1123, day 5: hESC n=3858, +YSLC CM n=3243, SOX1: day 1: hESC n=503, +YSLC CM n=735, day 3: hESC n=2933, +YSLC CM n=3023, day 4: hESC n=3426, +YSLC CM n=2507, day 5: hESC n=3764, +YSLC CM n=2620, n = 3 independent experiments each with one sample). (**E**) Diagram depicting the modified neural specification protocol in which YSLC conditioned media is used to replace the SB431542+NOGGIN treatment in the conventional protocol for 11 days, before culturing hESCs in the presence of just FGF2 for 4 days. (**F**) Immunofluorescence of SOX2 (green), SOX1 (grey), and a merge of both in hESCs cultured in mTeSR (hESC) and hESCs cultured in YSLC conditioned media (+YSLC CM) for the first 11 days of the neural protocol before being placed in FGF2 as outlined in panel E (scale bar = 50 μm). (**G**) Bar charts (± SEM) depicting RFI of SOX1 and SOX2 in cells from panel F. Mann–Whitney U-test; *p<0.05, ****p<0.0001 (number of cells analysed: SOX1: hESC n = 4949, +YSLC CM n=4736, SOX2: hESC n = 4947, +YSLC CM n=4736, n=3 independent experiments). (**H**) Relative expression levels (REL) (± SEM) of *PAX6, NESTIN*, and *NOTCH1* in hESCs cultured as outlined in panel E normalised to hESC control. Mann-Whitney U-test ; *p < 0.05 (n=4 samples, two independent experiments).

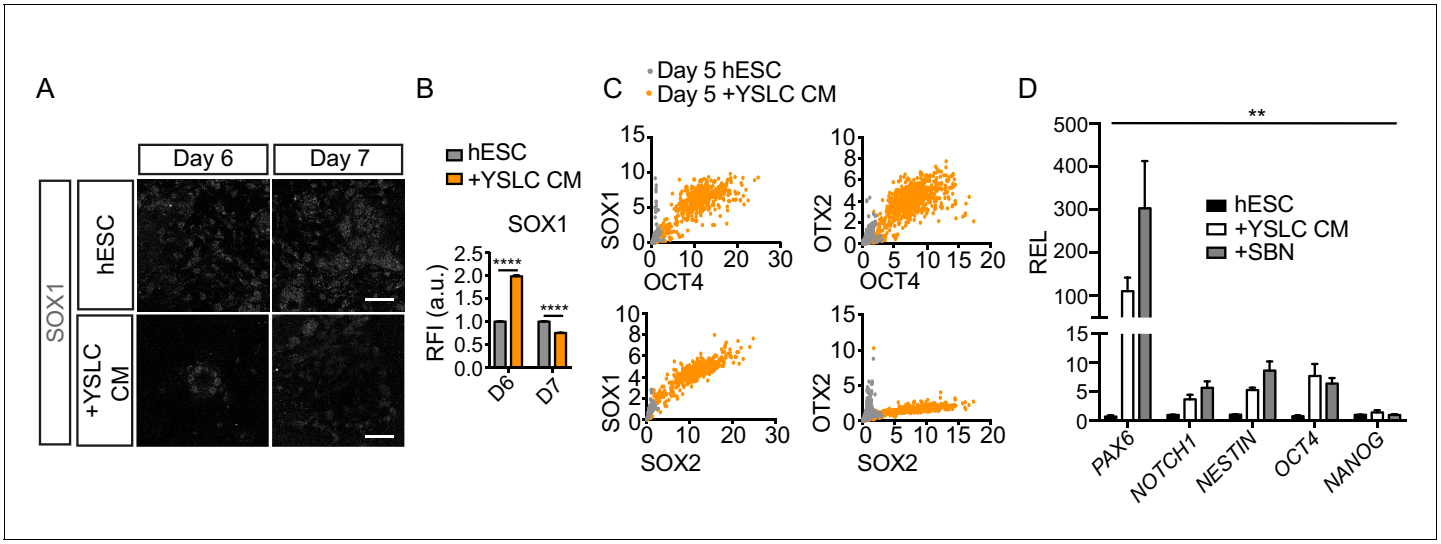

Figure 7—figure supplement 1. Yolk sac-like cells (YSLCs) induce the co-expression of anterior ectoderm markers and pluripotency markers in human embryonic stem cells (hESCs) in 2D and 3D. (**A**) Immunofluorescence of SOX1 (grey) in hESCs after 6 and 7 days cultured in mTeSR (hESC) or YSLC conditioned media (+YSLC CM) in 3D Geltrex (scale bar = 50 μm). (**B**) Bar charts (± SEM) depicting relative fluorescence intensity (RFI arbitrary units [a.u.]) of SOX1 in hESCs after 6 and 7 days cultured in YSLC conditioned media (+YSLC CM) relative to hESCs cultured in mTeSR (hESC) in 3D Geltrex. Two-way ANOVA with Sidak's multiple comparisons test comparing hESC VS +YSLC CM for each day. ****p<0.0001 (number of cells analysed day 6: hESC n=3338, + YSLC CM n=2205, day 7: hESC n=2745, + YSLC CM n = 4390, n=3 independent experiments). (**C**) Quantification of OTX2 or SOX1 vs. OCT4 or SOX2 at the single cell level in hESCs cultured in mTeSR (hESC) or YSLC conditioned media (+YSLC CM) in 3D Geltrex for 5 days based on protein immunostaining. Each dot represents an individual cell (numbers of cells depicted: SOX1vsOCT4: day 5 hESC n=1184, day 5 +YSLC CM n=478, OTX2vsOCT4: day 5 hESC n=1364, day 5 +YSLC CM n=748, SOX1vsSOX2: day 5 hESC n=1184, day 5+YSLC CM n=478, OTX2vsSOX2: day 5 hESC n=1364, day 5 +YSLC CM n=748). (**D**) Relative expression levels (REL) (± SEM) of *PAX6, NOTCH1, NESTIN, OCT4,* and *NANOG* in hESCs cultured as outlined in Figure 7E or in a dual SMAD inhibition neural protocol where SB431542 and NOGGIN (SBN) replace YSLC CM for the first 11 days of culture. Samples were normalised to hESC control. Two-way ANOVA with statistical result representing the p-value calculated when comparing columns for all genes (hESC vs. SBN vs. YSLC CM values); **p < 0.001 (n=4 samples, two independent experiments).